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J. Lewalter

N-Alkylvaline levels in globin as a new type of biomarker in risk assessment of alkylating agents

Abstract Adducts with the N-terminal valine of erythrocyte globin can serve as individual biomarkers of systemic and cellular exposure to endogenous and exogenous alkylating agents. In contrast to "detoxification markers" of this kind of mecapturic acids derived from alkylation of glutathione, individual N-alkylations of valine in globin reflect the formally "toxifying" part of the stress due to alkylating agents transformed into the ultimate toxicant. Thus, in contrast to the traditional methods of biological monitoring this approach enables a better evaluation of systemic exposure to reactive agents, adapted more sensibly to the exposure situation over the whole life span of erythrocytes, and it can serve as a specific biomarker of exposure for the purpose of health surveillance in occupational medicine. An individual evaluation of exposures in comparison with the range of corresponding background levels is discussed from the point of view of supplementary risk assessment in medical surveillance of occupationally exposed persons.

Key words Valine of erythrocyte globin · N-terminal valine · Edman degradation · thiohydantoins · N-methylvaline · N-cyanoethylvaline · N-benzylvaline · biomonitoring of acrylonitrile, benzyl chloride, hydrazine, and dimethyl sulfate · description of a case of acrylonitrile intoxication · possible exposure equivalents for acrylonitrile · ethylene oxide, and dimethyl sulfate

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J. Lewalter
BAYER AG, Department of Medicine, Building L 9,
D-51368 Leverkusen, Germany
Tel.: 049-214-3065679; Fax: 049-214-3021307

Introduction

Endogenous DNA methylations direct a wide variety of metabolic processes. In the erythrocytes they lead to the formation of a background level of up to 700 pmol N-methylvaline per g globin [9]. The physiological sources of endogenous methylation are S-adenosylmethione and formaldehyde, while exogenous methylations can be triggered by direct alkylating agents like methyl halides, dimethyl sulfate, dimethylformamide, diazomethane, styrene, etc., but also by indirect ones, such as dimethylnitrosamines, alkylmethylnitrosamines, or hydrazine [2, 3] (Fig. 1).

As can be expected, the indirect alkylating substances like hydrazine and the nitrosamines do not lead to the formation of N-methylvaline in erythrocytes. On the other hand, significant DNA methylation is induced in the liver by both direct and indirect alkylating agents [4]. The corresponding alkylations of N-terminal valine in erythrocyte globin are discussed as biological markers of cellular DNA alkylation [6]. This concept opens new prospects for risk prevention in occupational medicine backed up by ideas rooted in molecular biology [37].

Study objective

The objective of this study was to investigate whether in addition to endogenous alkylating agents, exogenous agents too, e.g. methyl halides, dimethyl sulfate, ethylene oxide, acrylonitrile, and benzyl chloride can alkylate the N-terminal valine of erythrocyte globin, with the ultimate aim of routine health surveillance in occupational medicine.

Material and methods

Design

N-alkylvaline values were determined in 500 persons who had been exposed to the above-mentioned alkylating agents and the results were compared with N-alkylvaline background levels in 30

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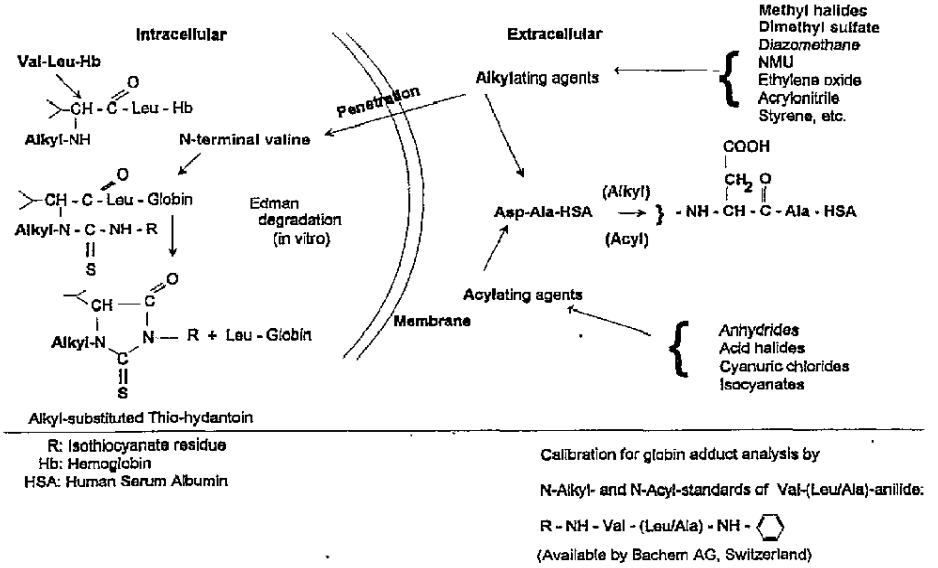

Fig. 1 Extracellular and intracellular alkylation and acetylation by direct reacting agents

unexposed persons. To demonstrate N-alkylation of N-terminal valine, globin was isolated from erythrocytes or from haemoglobin and separated by Edman degradation; the resulting thiohydantoin derivatives were then quantified by gas chromatography/mass spectroscopy (GC/MS) [8, 9].

Chemicals and reagents

All chemicals were of the best available analytical grade and were obtained from Merck (Darmstadt, Germany) or from Sigma (Deisenhofen, Germany), except for formamide (ultrapure, USB, Bad Homburg, Germany) and pentafluorophenyl isothiocyanate (Fluka, Buchs, Switzerland). N-Methyl-, N-hydroxyethyl-, and N-cyanoethylvalineleucine-anilides (purity > 98%, determined by thin-layer chromatography) were synthesized and specified by Bachem AG (Bubendorf, Switzerland).

Biological samples and isolation of globin

Whole blood (5 ml) was collected from each person by venipuncture in EDTA-containing evacuated collection tubes (Vacuejects from Sherwood Medical, Crawley, Sussex, UK) and was centrifuged for 5 min at 1200 g. The supernatant plasma was discarded.

The erythrocytes were washed with three 2.5 ml portions of 0.9% saline and centrifuged for 10 min at 1200 g. Haemolysis was achieved by adding 2.5 ml of ice-cold distilled and deionized water and keeping the samples at –20°C overnight. Cell membrane fragments were then removed by centrifugation for 30 min at 5°C and 30,000 g. To prepare about 150 mg globin from a sample, 2 ml of each haemolysate was added to 10 ml of 50 mM hydrochloric acid in aqueous 2-propanol [4 ml conc. HCl (50 mmol) made up to 1 l with 2-propanol]. The samples were stored for at least 1 h at 4°C and subsequently centrifuged for 10 min at 2000 g.

7 ml ethyl acetate was added to the supernatant and the solution was stored at 4°C overnight. The precipitated globin was spun down for 5 min at 2000 g, washed with four 10 ml volumes of ethyl acetate, and centrifuged for 5 min at 1500 g. Finally the globin was washed with 5 ml of n-hexane and dried in a vacuum desiccator.

Edman degradation

All analyses were carried out in duplicate, and with each batch of samples a control globin from unexposed individuals was analysed. The N-alkylated amino acids were analysed by gas chromatography/mass spectrometry (GC/MS) following a slightly modified Edman procedure as described by Törnqvist et al. [8]. Briefly, 100 mg of each globin sample was dissolved in 1.5 ml of formamide solution, sonicated for 15 min until a clear solution had been obtained, and 30 µl of 1N NaOH and 30 µl of internal standard (47 µg N-benzylvaline per litre of blood, made by diluting a solution of 19.1 mg N-benzyl-Val-Leu-anilide in 100 ml ethanol in the ratio of 1:100 with ethanol) was added, followed by 10 µl of pentafluorophenyl isothiocyanate. The samples were rotated at room temperature and incubated for 90 min at 45°C. The solutions were extracted with two 2.5 ml portions of diethyl ether and centrifuged for 1 min at 10°C and 3500 g each time. The combined organic phases were evaporated to dryness in a vacuum centrifuge at 200 mbar and 40°C over about 30 min and the residues were dissolved in 500 µl toluene. The samples were washed with 2 ml of distilled water and subsequently with 2 ml of freshly prepared 0.1 M sodium carbonate. The toluene layer was evaporated in a vacuum centrifuge at 20 mbar and 40°C over 80 min and the residue was redissolved in 50 µl toluene. 1 µl of each sample was finally analysed by GC/MS.

Quantitation of globin adducts

1 µl of each toluene solution was injected splitlessly into the gas chromatograph. The pentafluorophenyl thiohydantoins of N-methylvaline, N-hydroxyethylvaline, N-cyanoethylvaline, and N-benzylvaline were identified by their retention times and quantified by two characteristic masses: m/z 310/338 for N-methylvaline, m/z 308/350 for N-hydroxyethylvaline, m/z 335/377 for N-cyanoethylvaline, and m/z 297/414 for N-benzylvaline. The amounts of N-methylvaline, N-hydroxyethylvaline and N-cyanoethylvaline in the globin samples were calculated from calibration curves.

GC/MS conditions

Hewlett Packard 5890 series II gas chromatograph: Fisons fused silica DB-5MS capillary column, 25 m × 0.22 mm, 0.33 µm film

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thickness; carrier gas helium 5.0; column pressure: 9 psi; temperatures: injector 280°C, column 120°C, rising at 15°C/min to 220°C and then at 5°C/min to 280°C, transfer line 300°C, ion source 200°C, quadruple 100°C; ion source pressure 2×10^{-6} torr; ionization mode electron impact (EI), electron energy 70 eV; detection mode selected ion monitoring (SIM), dwell time 100 ms/ion trace, electron multiplier 2800 V.

Calibration and validation of the method

Calibration lines for the selected N-alkylvalines were constructed in the range of 1 to 400 µg/l blood. Briefly, a stock solution with a concentration of 10 mg of each N-alkylvaline per 100 ml ethanol was prepared by dissolving 24.4 mg N-methyl-Val-Leu-anilide, 21.6 mg N-hydroxyethyl-Val-Leu-anilide, and 21.1 mg N-cyanoethyl-Val-Leu-anilide in 100 ml ethanol. Dilution of this stock solution with methanol in the ratio of 1:10, 1:100, and 1:1000 yielded three standard solutions. These were used to spike 100 mg samples of pooled globin, isolated as mentioned above, and dissolved in 1.5 ml of formamide with 1 to 100 ng of each of N-alkyl-Val-Leu-anilide per sample. The samples were next mixed with the internal standard and with isothiocyanate and worked up as described above. The area integrals of the internal standard (N-benzylvaline) were compared with those of the final recovery standard (leucine) to evaluate the reaction and extraction efficiency of the method. The ratios of the area integrals of each N-alkylthiohydantoin to N-benzylthiohydantoin were plotted against the concentration of corresponding N-alkylvaline liberated from the globin adduct per litre of blood.

Statistical analyses

The exposure data for ethylene oxide, acrylonitrile and dimethyl sulfate and for their globin adducts did not depart significantly from a normal distribution, and logarithmic transformation of the data yielded a still better approximation to the normal distribution. Both arithmetic and geometric means were calculated. All parameters of the different exposure groups were compared by two-way analysis of variance.

Simple and multiple regression was used to study the relations between exposure to ethylene oxide, dimethyl sulfate, or acrylonitrile and the levels of their adducts found in the globin samples.

The statistical analyses were done with the aid of NCSS software, using the DOS version 5.

Results

The background levels of methylation and hydroxyethylation of N-terminal valine in globin reported in the literature were reproduced, and cyanoethylation was demonstrated for the first time [5]. These are usually the consequence of endogenous (S-adenosylmethionine), but especially also of (ubiquitous) exogenous stress by confounding factors like smoking etc. Elevated N-alkylvaline levels in erythrocytes can also be observed as a result of industrial exposure to substances at the workplace, and particularly after accidental intoxications (Fig. 2).

Experience has been obtained in particular for the following areas under investigation:

1. Occupational health surveillance in a hydrazine plant: at three successive biological monitoring examinations no N-methylvaline formation in erythrocytes exceeding the individual background level was observed (plant H).

2. Occupational health surveillance of persons working under conditions of regular exposure to monochloromethyl ether: elevated N-methylvaline formation could not be demonstrated in erythrocytes (plant L).

3. In some persons handling acrylonitrile (routine occupational health surveillance and intoxications) measurable cyanoethylvaline formation in globin has been observed, in general correlating well with cyanoethylmercapturic acid excretion in urine (plant D).

4. Surveillance of persons handling various alkylating agents, e.g. methyl halides, dimethyl sulfate, acrylonitrile, and ethylene oxide, yielded predominantly reference values (Fig. 3) and occasional significantly elevated conjugates with the N-terminal valine in erythrocytes globin. The extent of conjugate formation generally correlated with the extent of external contamination (contaminated skin area or duration of inhalation) (plants K and Z).

Fig. 2 N-Alkylvaline levels in the globin of workers routinely handling alkylating substances in 8 h shifts (selections from routine and special surveillance in 1990–1995)

N-Alkylvaline (Background levels in µg/l blood)	Plants	Persons	N-Alkylvaline values in µg/l blood	IgE-levels (U/l) total	IgE-levels (U/l) specific
N-Methylvaline					
(S : 8,5 (5,1 - 11,4))					
(NS: 7,0 (3,5 - 10,5))	L	183	S/NS:10 ± 2,0	36 (<5-190)	1,2 (0,5-1,7)
(IgE-total : < 78 PU)*					
(IgE-Methyl-HSA: < 1 RU)*	P	74	S/NS:24 ± 5,5	59 (<5-230)	1,6 (0,5-2,3)
N-Hydroxyethylvaline	Uni-Hospital	27	S/NS: 340 (10-1100)	110 (20-570)	2,0 (0,9-4,2)
(S :4,0 (< 2 - 14))	K	150	S/NS: 43 (25-60)	65 (7-250)	1,7 (0,4-2,5)
(NS:< 2,0)	Z	220	S/NS: 40 (23-57)	71 (9-210)	1,9 (0,7-2,8)
(IgE-Hydroxyethyl-HSA: < 1RU)*					
N-Cyanoethylvaline	D	67	S : 13 (5-20)	35 (14-230)	1,7 (0,5-2,4)
(S :4,0 (0,8-9,2))			NS : 8 (2-12)		
(NS:< 0,5)	K	155	S : 11 (6-15)	47 (21-270)	1,5 (0,4-2,5)
(IgE-Cyanoethyl-HSA: < 1 RU)*			NS : 6 (2-10)		
N-Benzylvaline					
(S: < 1)	N	74	S/NS : 19 (4,4 - 42,8)	74 (34-520)	1,9 (0,9 - 5,7)
(NS: < 1)	O	111	S/NS : 7 (1,3 - 24,5)	43 (30-350)	1,4 (0,6 - 3,1)
(IgE-Benzyl-HSA: < 1 RU)*					

*) 95. Percentile
S = Smoker
NS = Non-Smoker

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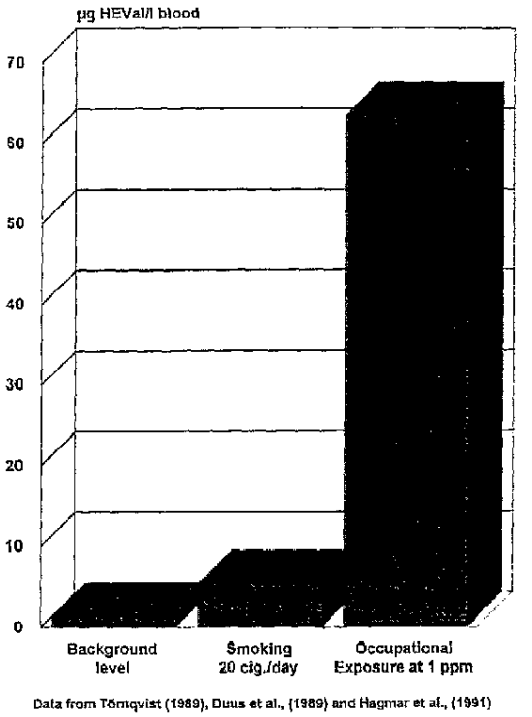


Fig. 3 Cumulative levels of hydroxyethylvaline (HEVal), a product of ethylene oxide, in haemoglobin

5. In one plant, where esters of inorganic acids are handled, N-methylvaline levels sometimes exceeding the background factor of 2 were found in erythrocytes in surveillance investigations and in special investigations based on specific types of work. It was found that this elevated N-methylation of valine was caused by the handling of trimethyl phosphite (plant P).

From the standpoint of molecular biology, N-alkylation of valine by exogenous alkylating agents is understood to be a consequence of competitive reactions with electrophilic substrates. The "toxifying" N-alkylation of macromolecules exceeds individual background levels only when the local availability of "detoxifying" substrates like gluta- [redacted] activity of the corresponding enzymes is in- [redacted]

Possible exposure equivalents for ethylene oxide

Working materials which are justifiably suspected of having carcinogenic potential, like ethylene oxide, when examined in biological tests, will not be considered here from the standpoint of the strict BAT value definition of the German MAK Commission, but will rather be regarded in the light of industrial-medical experience for demonstration and quantification of individual stress due to the compound. If the concentrations of this substance or of its metabolites in biological material exceed those expected on the basis of the substance concentration in air at the workplace, then some additional absorption – normally percutaneous – must have taken place [20].

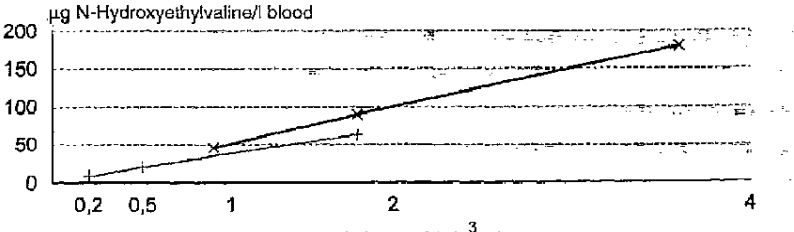
This biomonitoring in parallel with the annual health surveillance examinations of workers handling ethylene oxide permits us to determine the correlation between ethylene oxide concentration in the workplace atmosphere and the concentration of ethylene oxide-globin adducts in the erythrocytes of individual blood samples. From these extensive exposure equivalents for ethylene oxide it can be established what internal stress is caused by inhalation of ethylene oxide alone [2, 41] (Fig. 4).

Description of a case of intoxication with acrylonitrile

The essential significance of glutathione-transferase in detoxification of stress due to alkylating agents is well illustrated by a case concerning acrylonitrile (Fig. 5).

Acrylonitrile is a liquid monomer commonly used in polymer chemistry for the production of various copolymers, and is well absorbed both by inhalation and percutaneously. In acute acrylonitrile intoxications certain clinical signs are observed which point to a strong stimulation of the parasympathetic nervous system: vasomotor disturbances, hypersalivation, nausea, vomiting, headaches, and spasms have been documented.

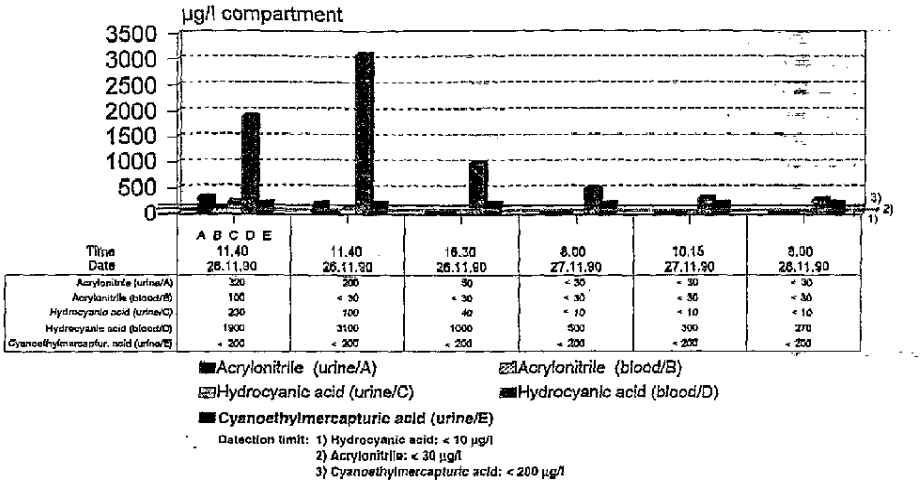
Fig. 4 EKA values established for ethylene oxide (EO) handling in the workplace



Air mg EO/m³	0,2	0,5	0,92	1,83	3,66
EKA-values (µg/l blood) Calibration according to x Törnqvist + Lewalter [persons]	8 (3 - 27) [216]	20 (9-38) [132]	45	90 63 (45-87) [14]	180

Source: https://www.industrydocuments.ucsf.edu/docs/qynx0001

Fig. 5 Acrylonitrile intoxication



	Minimal values	Maximal values	Average values	
Blood	in µg/l blood		S	NS
Acrylonitrile	< 10	80	30	
Hydrocyanic acid: 100. percentile	60	470	220	140
95. percentile	70	170	180	110
N-Cyanoethylvaline	< 1	27	14	8
Urine	in µg/l urine		S / NS	
Acrylonitrile	< 30	500	120	
Rhodanide	2000	7000	5000	
Cyanoethylmercapturic acid	<200	37000	10000	

AM = Ambient Monitoring
S = Smoker
NS = Non-Smoker

Fig. 6 Acrylonitrile biomonitoring. AM: < 1/4 TRK (< 3 mg/m³); *n*: 28 persons

In the case described here a technician ignoring the operating instructions accidentally sat for 3–4 h in a puddle of an acrylonitrile-containing liquid before complaining of nausea and headache. He vomited at the place of work and in our first-aid station he was treated intravenously with Fluimucil. Shortly afterwards he was free from symptoms. After about one hour biomonitoring of his blood and urine showed, apart from a slight acrylonitrile stress, an HCN level of at first 1.9 mg and after approximately 3 h one of 3.1 mg/l blood (Fig. 5).

In view of this, the patient was also given an intravenous injection of 4-dimethylaminophenol (DMAP). The methaemoglobin rose to 24% and the cyanide level went down within 1.5 h to 1.0 mg/l blood, falling after 16 h to 0.5 mg/l.

The patient himself did not notice any adverse influence of the critical rise in hydrocyanic acid. He is a heavy smoker, with an individual reference value of approximately 250 ± 20 µg cyanide per litre of blood, a level exceeding the cyanide background of smokers by about 20–30%. No significant excretion of cyanomercapturic acid could be demonstrated.

Biomonitoring of acrylonitrile

After this observation of insufficient acrylonitrile metabolism 28 further persons who regularly handle acrylonitrile were surveyed, monitoring both acrylonitrile and the corresponding hydrocyanic acid levels. It was found that in approximately 95% of these subjects the HCN reference values corresponded to the level usual in smokers. In the remaining 5%, however, the hydrocyanic acid background exceeded the usual smoker level of about 200 µg/l blood by more than 100% (Fig. 6).

These values were confirmed by a second test, in which hydrocyanic acid was determined in the blood of 360 non-smokers: again approximately 5% of this group showed HCN levels clearly above the background in the blood of smokers (Fig. 7).

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Hydrocyanic acid µg/l blood	N-Cyanoethyl-Valine µg/l blood	Acrylonitrile µg/l urine	Persons n	% of group
≤ 50	1,7 (1-3)	< 10	140	39
> 50 ≤ 150 (± 50)	2,1 (1-3)	< 10	201	56
> 200	7,3 (>3-9)	< 10	19	5
total			360	100

Fig. 7 Biomonitoring of non-smokers not handling acrylonitrile, classified by their hydrocyanic acid levels in blood (95th percentile of average values)

Figure 8 shows an overview of the metabolism of acrylonitrile. Part of the internal acrylonitrile dose is obviously exhaled in an unchanged form or is excreted with urine. A further part of the acrylonitrile is conjugated enzymatically or non-enzymatically with glutathione. This conjugate is the source of the N-acetyl-S-(2-cyanoethyl)cysteine (cyanoethylmercapturic acid) which appears in the urine. Part of the incorporated acrylonitrile penetrates the cell membranes and can here, for example in the erythrocytes, react with the N-terminal amine group of valine to form cyanoethylvaline (Fig. 1).

Finally a small fraction of the acrylonitrile dose is oxidized to epoxide. The nitrile group is then split off as hydrocyanic acid and eventually excreted in urine as thiocyanate [1].

This epoxide pathway has been identified in animal studies, and was later confirmed by the metabolite pattern in some acrylonitrile-stressed men. Individual differences in predisposition emerge from differences in the isoenzyme profile of GSH-transferase activity as well as from the acrylonitrile metabolites in urine and variations in N-cyanoethylvaline levels in erythrocyte globin (Fig. 7 and 8).

It remains to be noted that in some 5% of the investigated persons acrylonitrile is not "detoxified" in the organism by the formation of mercapturic acid because of inactivation of a specific glutathione transferase. Instead, it is actually "toxified" by multifunctioning oxidases via epoxidation (Fig. 8).

Apart from the acrylonitrile, hydrocyanic acid can be used as a biomarker for the very reactive intermediate formed during the GSH-deficient metabolism of acrylonitrile.

Fig. 8 Metabolism of acrylonitrile (according to Ahmed and Trieff, 1983)

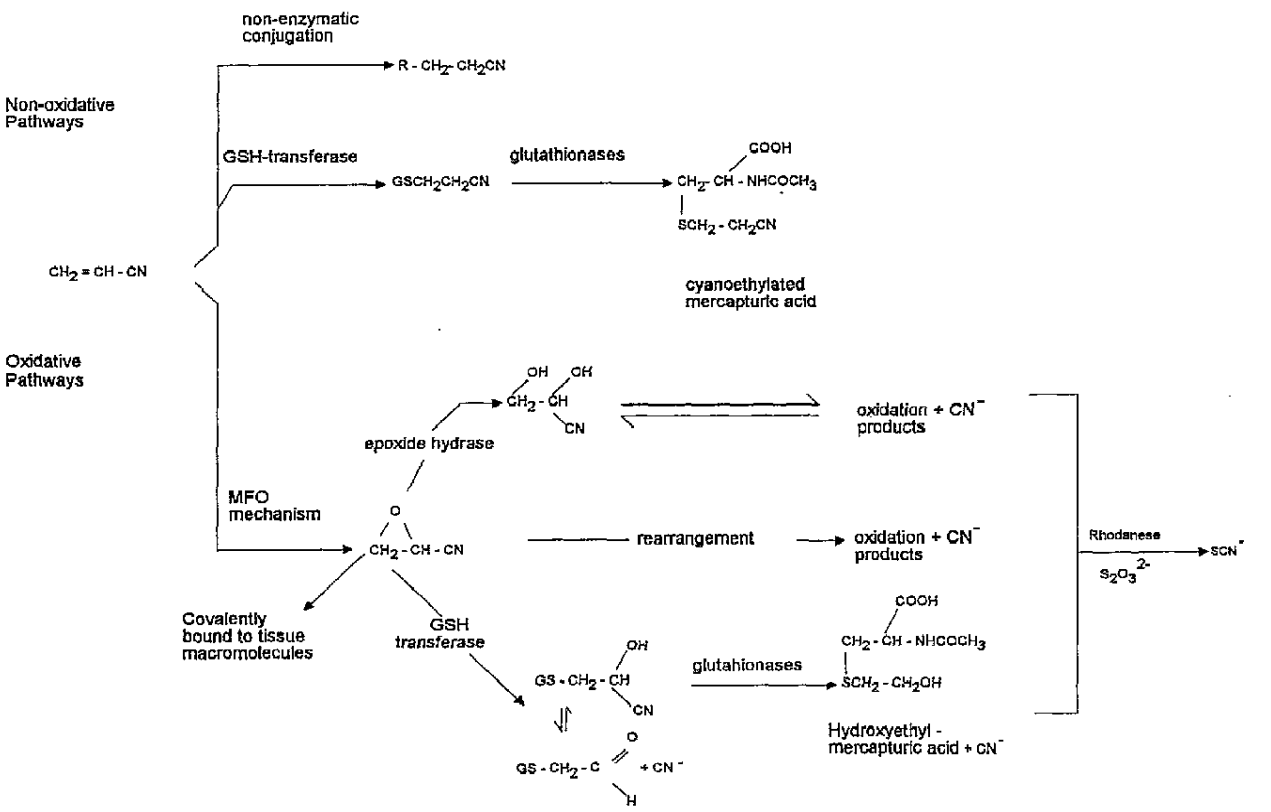

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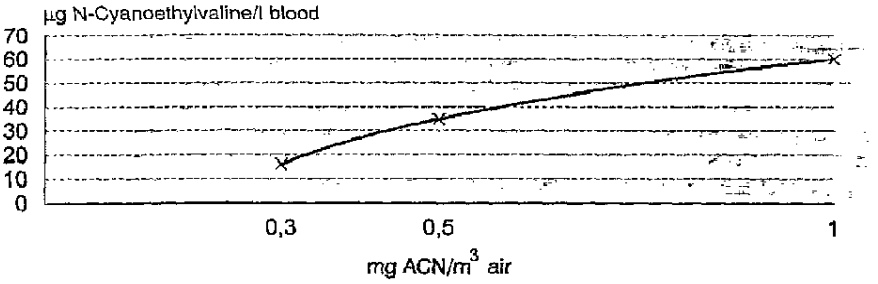
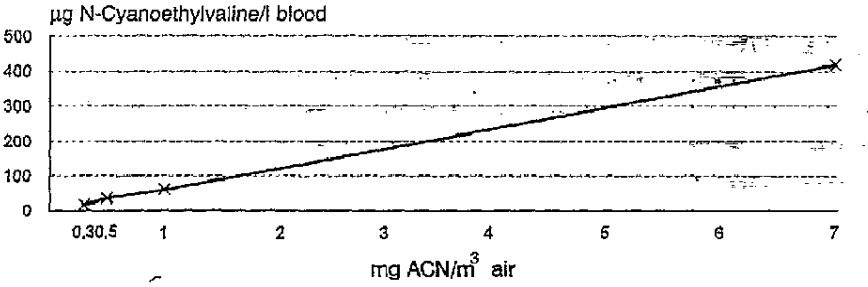
Fig. 9 EKA proposal for acrylonitrile (ACN) handling on the basis of adducts with N-terminal valine in globin (directly without linear regression)



Air mg ACN/m^3	0,3	0,5	1,0	(7,0) (extrapolated by linear regression)
possible EKA values*) µg N-cyanoethyl-val/l blood	16 (6-33)	35 (12-56)	60 (23-95)	(420)
[persons]	[438]	[110]	[43]	

*) Calibrated on the N-cyanoethyl-val-leu-anilide standard

Fig. 10 EKA proposal for acrylonitrile (ACN) handling on the basis of adducts with N-terminal valine in globin (extrapolation by linear regression)



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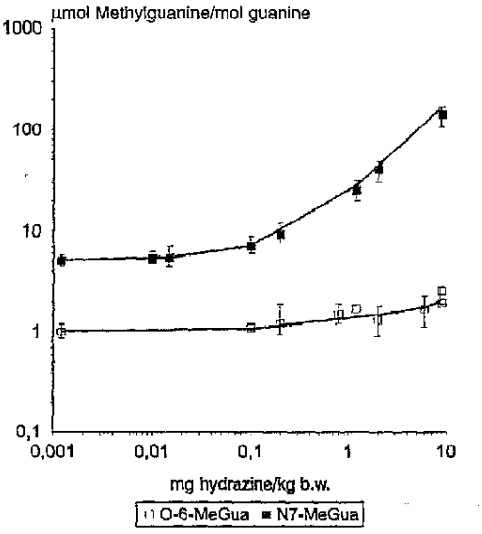
[persons]	[438]	[110]	[43]	

*) Calibrated on the N-cyanoethyl-val-leu-anilide standard

Dose mg hydrazine per kg rat	µmol N-7-Methylguanine per mol guanine	µmol O^6-Methylguanine per mol guanine	Ratio N-7- to O^6-Methylguanine (Swain-Scott-factor)
90 (LD50)	869	58	15 : 1
45	421	15	28 : 1
0	56	< 1	> 56 : 1

Fig. 11 Methylation of liver DNA guanine after administration of various doses of hydrazine in rats (Becker et al. 1981)



Formation of N-7- and O-6-Methylguanine (MeGua) in liver DNA of rats exposed to hydrazine. N-7-Methylguanine was detected by GC/MS (Leverkusen) und 0-6-Methylguanine by RIA (Essen).

Fig. 12 Hydrazine experiments – Dose response curve

Possible exposure equivalents for acrylonitrile

Biomonitoring of acrylonitrile-handling workers in parallel with the annual health surveillance examinations permits a direct determination of the correlation between the acrylonitrile concentration in the workplace atmosphere and the concentration of N-cyanoethyl adducts with globin in the erythrocytes of individual blood samples [5] (Fig. 9).

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Fig. 13 Correlation between atmospheric hydrazine and the consequences in various biological compartments in 4 workers at a hydrazine plant in comparison with controls (Me-Gu = methylguanine)

Persons	Smoking habits S: smoker NS: non-smoker	Air Hydrazine µg/m3	Urine Hydrazine µg/l	Urine N-Acetyl-hydrazine µg/l	Globin N-Methyl-Valine µg/l blood	Globin N-Cyanoethyl-Valine µg/l blood	Lymphocytes O6-Me-Gu µmol/mol guanine	Lymphocytes N-7-Me-Gu µmol/mol guanine
2/post shift	S	30,0	40,0	< 30	11,5	7,1	< 1	84,0
2/pre shift	S	J.	80,0	60,0	10,8	7,0	< 1	79,0
12/post shift	NS	13,0	20,0	< 30	9,3	1,5	< 1	3,8
12/pre shift	NS	J.	25,0	40,0	9,4	1,5	< 1	2,8
17/post shift	S	34,0	200,0	35,0	11,7	7,5	< 1	95,0
17/pre shift	S	J.	30,0	40,0	11,9	7,7	< 1	89,0
23/post shift	NS	10,0	45,0	< 30	10,5	1,0	< 1	6,4
23/pre shift	NS	J.	10,0	< 30	10,5	1,1	< 1	4,6
Controls (50 unexposed S and NS)	S	J.	< 10	< 30	11,0 ± 2,6	7 ± 2	< 1	69,7 ± 14,6
	NS	J.	< 10	< 30	10,7 ± 2,5	2 ± 1	< 1	1,7 ± 1,0

Hours after the accident	Gobin N-Methylvaline µg/l blood	Lymphocytes N-7-Methyl-guanine µmol/mol guanine	Urine 3-Methyladenine µg/g creatinine
+ 1 h	8,5	4,0	9,0
+ 3 h	10,3	5,0	27,0
+ 7 h	12,5	5,0	31,0
+ 24 h	12,0	4,0	12,0
+ 48 h	12,3	7,0	8,0

Fig. 14 Dimethyl sulfate – intoxication

Extrapolation to the threshold limit value by linear regression gives Fig. 10.

Biomonitoring of benzyl chloride

The preliminary results of biomonitoring workers routinely handling benzyl chloride are shown in Fig. 2. The corresponding external air benzyl chloride exposure was at all times below 1/10 of the threshold limit of 5.2 mg/m^3.

Biomonitoring of hydrazine

With indirect alkylating agents such as hydrazine and the nitrosamines a dose-dependent DNA alkylation takes place, as has been demonstrated in animal studies for hydrazine, first at high doses (Fig. 11) and later also at low doses (Fig. 12).

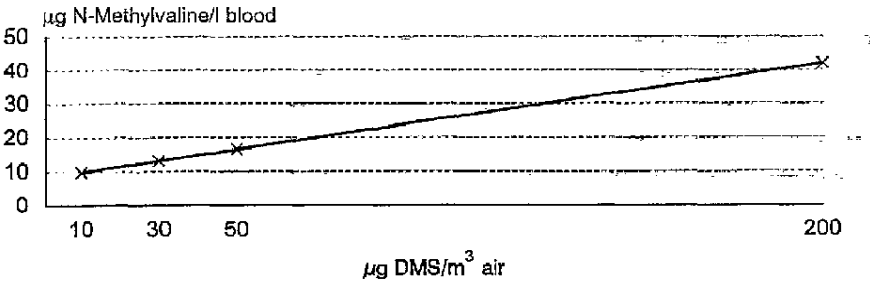
With hydrazine doses of more than 0.1 mg/kg in rats only a slight formation of O^6-methyl-guanine was observed, alongside significant N-7-methyl formation. With hydrazine doses smaller than 0.1 mg/kg no additional DNA methylation was observed in comparison with untreated animals (background level) (Fig. 12).

Meanwhile, these associations were also found in human subjects and in occupational-medicine surveys (Fig. 13).

It is worth noting that hydrazine did not cause any significant methylation of protein in the erythrocytes. Hydrazine-induced adduct formation was only detected in DNA monitoring and not in the Hb monitoring frequently recommended as a "correlate".

The metabolism of hydrazine e.g. in man shows the necessity to differentiate the resulting DNA adducts into critical, probably genotoxic, O^6-methylation and the ap-

Fig. 15 EKA proposal for dimethyl sulfate (DMS) handling on the basis of adducts with N-terminal valine in globin



Air µg DMS/m^3	10	30	50	200 (extrapolated by linear regression)
possible EKA values*) µg N-Methyl-Val/l blood [persons]	9,7 (4,2-15,8) [24]	13,1 (6,8-19,1) [19]	16,5 (8,2-22,7) [7]	(42)

*) Calibrated on the N-methyl-val-leu-anilide standard

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The reference value concept in the prevention of occupationally exposed workers

Before the scientific evaluation of practical biological tolerance values for working substances is carried out, it is advisable to monitor the known background and reference values or the detection limits of the incorporated working substances or their metabolites, adducts or conjugates with GSH (urine), HSA (serum), Hb or globin (RBC) and DNA (lymphocytes) based on a validated analytical procedure in order to detect and to assess the individual handling risk, particularly in connection with carcinogenic or teratogenic substances.

In obscure or dubious cases the biomonitoring of conjugates or adducts of working substances with macromolecules should be preferred because this permits the monitoring of individual metabolic capacity as well as the proportion of incorporated active substances, that is excreted neither directly nor unchanged (40).

Fig. 16 The reference value concept in the protection of occupationally exposed workers (J. Lewalter 1993)

parently non-critical N-7-methylation of guanine. The screening methods used for DNA adduct formation, like the post-labelling technique or the determination of thioethers in urine, will inevitably lead to incorrect conclusions [7, 14].

The enzymatic and immunological consequences of various DNA structures associated with corresponding activities of repair enzymes and oncogenes, as well as immunosuppressive effects, can only be mentioned briefly here. The increasingly more efficient repair enzymes observed in the presence of reduced hydrazine stress lead to an increasing ratio of N-7-methyl "doses" to O^6-methyl "effects", the so-called Swain-Scott factor. This may be an indicator for the existence of a "natural" tolerance level for hydrazine [4] (Fig. 11).

Biomonitoring of dimethyl sulfate

The extent of N-alkylation of valine by indirect alkylating agents is determined by the nature and localization of their activation in the organism. Direct alkylating agents like dimethyl sulfate induce very strong N-methylation of the N-terminal valine of erythrocyte globin (Fig. 14), whereas indirect alkylating agents, like hydrazine (Fig. 13), do not show this effect.

Possible exposure equivalents for dimethyl sulfate

Biomonitoring of dimethyl sulfate-handling workers in parallel with the annual health surveillance examinations permits a determination of the correlation between the dimethyl sulfate concentration in the workplace atmosphere and the concentration of N-methyl-globin adducts in the erythrocytes of individual blood samples [9] (Fig. 15).

Discussion

Among the working substances investigated, N-hydroxyethyl-, N-cyanoethyl-, and N-methylvaline levels significantly above the individual backgrounds were found after exposures within the atmospheric TLV only in workers handling ethylene oxide, acrylonitrile, and dimethyl sulfate.

Stress due to accidental situations with a variety of alkylating agents in general led to increased N-alkylation of N-terminal valine, in some cases to levels 10–100 times the corresponding background. Individual differences in the level and apparently also the half-life of N-alkylvaline have been observed for the background levels, and particularly also for exogenous stress situations.

In terms of molecular biology direct alkylation of N-terminal valine in globin appears to be plausible, especially since in general corresponding mercapturic acids resulting from alkylation of glutathione can also be demonstrated.

Indirect methylation e.g. by exogenous induction of the endogenous C1-methylation pool may occur, but is not very probable. In the case of hydrazine this effect in particular would be inhibited. On the other hand, the missing methylation of globin in hydrazine metabolism could confirm the postulated intermediate participation of diazomethane. The highly reactive diazomethane might be used up completely at the place of its production (the liver).

Furthermore from molecular biology investigations and from mutagenicity studies it is known that alkylating agents can lead to alkylation of DNA and in particular to the formation of O^6-alkylguanine. Here they compete with DNA-relevant physiological metabolic changes, which are known to be regulated by endogenous methylations.

In general, the hypermethylation of nucleic acids is compensated by a powerful capacity for DNA repair. This ensures lifelong prevention of endogenous and exogenous methylation damage to the DNA [2, 3, 6, 7].

At present few German BAT and EKA and American BEI values are available. Until the evaluation of biological tolerance thresholds derived from actual practice in occupational medicine, toxicology, and epidemiology is carried out, it is recommended from the point of view of preventive medicine to supplement the occupational health surveillance by the concept of risk assessment based on individual background values (Fig. 16), especially as in the meantime many validated reference values are becoming available [40].

The following tables, classified according to the German MAK concept, provide an overview of the current knowledge and availability of reference values for substances of interest in health surveillance monitoring and in the risk assessment of occupationally exposed persons (Table 1).

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Table 1 Reference values and values derived from experience in occupational medicine (AEW), as well as biological tolerance values for working substances according to the German MAK list, Part III A 1: working substances which have been unequivocally proved to be carcinogenic, which can be supervised by quantitative determination in biological materials

Working material	Parameter	Biological Material	Methode	Reference values Averge values	Limiting Values	Occupational medical experienced values	Tentative maximum permissible concentration EKA-value*)	References
4-Aminobiphenyl	4-aminobi-phenyl	Urine Blood Hemoglobin	GC-ECD/MS; HPLC-ECD		100 ng/l 100 ng/l S :70 ng/l Blood NS:20 ng/l Blood	< 1,0 µg/l < 0,1 µg/l Blood		4,10,12,13 24,26 27,31,32 35,36,37 14
Arsentrioxid, Arsenpent-oxid, arsenic acid and his salts	arsenic	Urine	Hydrid-AAS	3 - 5 µg/g Creat.	< 40 µg/g Creat.		130 µg/l	23,30
Benzidine and his salts	benzidine	Urine Blood Hemoglobin	GC-ECD/MS; HPLC-ECD		< 25 ng/l < 25 ng/l S : < 5,0 ng/l Blood NS:< 5,0 ng/l Blood	< 1,0 µg/l < 0,1 µg/l Blood		4,24,26 27
Benzene	benzene	Blood	Head space	S: 500 ng/l NS: 200 ng/l	S : 901 ng/l NS:576 ng/l		5,0 µg/l	11,17,19
	phenol	Urine	HPLC	3 - 5 mg/l	20 mg/l		45 µg/g Creat.	18,19
	S-phenylmer-capturic acid	Urine	GC-MS		< 4,0 µg/l			
	trans,trans-muconic acid	Urine	HPLC		< 0,2 mg/l	(Depression by toluene!)	2,0 mg/l	21
	hydroquinone	Urine	GC-ECD		< 2,0 mg/g Creat.			
4-Chloro-o-toluidine	4-chloro-o-toluidine	Urine Blood Hemoglobin	GC-ECD/MS; HPLC-ECD		< 100 ng/l < 50 ng/l S :< 25 ng/l Blood NS:< 25 ng/l Blood	< 1,0 µg/l < 0,1 µg/l Blood		20,37
2-Naphthylamine	2-naphthyl-amine	Urine Blood Hemoglobin	GC-ECD/MS; HPLC-ECD		50 ng/l 50 ng/l S :20 ng/l Blood NS:10 ng/l Blood	< 1,0 µg/l < 0,1 µg/l Blood		4,24,26 35
Nickel (soluble compounds)	nickel	Urine Blood Plasma	GF-AAS GF-AAS	2,0 - 5,0 µg/l 0,2 - 0,5 µg/l 0,2 - 0,5 µg/l	10,0 µg/l 1,0 µg/l 1,0 µg/l		45 µg/l	15,22,29 18,28,33
Vinyl chloride	thiodiglykolic acid	Urine Blood	GC-MS Head space		< 10 µg/l		2,4 mg/ 24 Std.	6,13,34
Zinkchromate	chromium	Urine Blood RBC	GF-AAS GF-AAS GF-AAS		< 1,0 µg/g Creat. < 1,0 µg/l < 1,0 µg/l Blood	< 10 µg/l < 10 µg/l < 5,0 µg/l Blood	40 µg/l 35 µg/l Blood	4,20,25,37

EKA: exposure equivalents for carcinogenic working materials in the German list of MAK and BAT values.

Conclusions

1. Adducts with the N-terminal valine of erythrocyte globin can serve as individual biomarkers of systemic and cellular exposure to endogenous and exogenous alkylating agents.

2. In contrast to "detoxification markers" of this kind of mecapturic acids derived from alkylation of glutathione, individual N-alkylations of valine in globin reflect the formally "toxifying" part of the stress due to alkylating agents transformed into the ultimate toxicant.

3. In contrast to the traditional methods of biological monitoring this adduct approach enables an evaluation of the systemic exposure to reactive agents adapted more adequately to the exposure situation during the entire life span of the erythrocytes, and it can serve as a specific biomarker of exposure for the purposes of health surveillance in occupational medicine.

4. In toxicological risk assessments alkylation of N-terminal valine is sometimes taken as an effect marker of corresponding DNA alkylation. In the evaluation of haemoglobin adducts in occupational medicine it must be taken into consideration that this alkylation may formally appear to be higher, owing to the accumulation of numerous minimal stresses, than would be expected from DNA conjugations due to the actually effective stresses alone.

5. For the formation of N-alkylvaline caused by some chemicals the range of the individual and group-related background levels could be evaluated. Corresponding stress of cellular DNA metabolism is in general only expected if the N-alkylvaline adducts exceed the background level by a factor of 10–100.

6. It should be borne in mind that the molecular-biology connections can only be interpreted as an occupational health hazard exposing the individual metabolisms to stress when a significant elevation above the individual

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background can be demonstrated for particular cells after alkylation of specific DNA loci, with a concurrent excessive demand on cell repair activity. There is a further limitation on deductions concerning the extent of corresponding DNA alkylation from the N-alkylvaline adduct levels in haemoglobin, since these levels are in general controlled by highly efficient repair enzyme systems.

7. An individual evaluation of exposure values in comparison with the range of the background levels is discussed from the standpoint of supplementary risk assessment in medical surveillance of occupationally exposed persons.

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